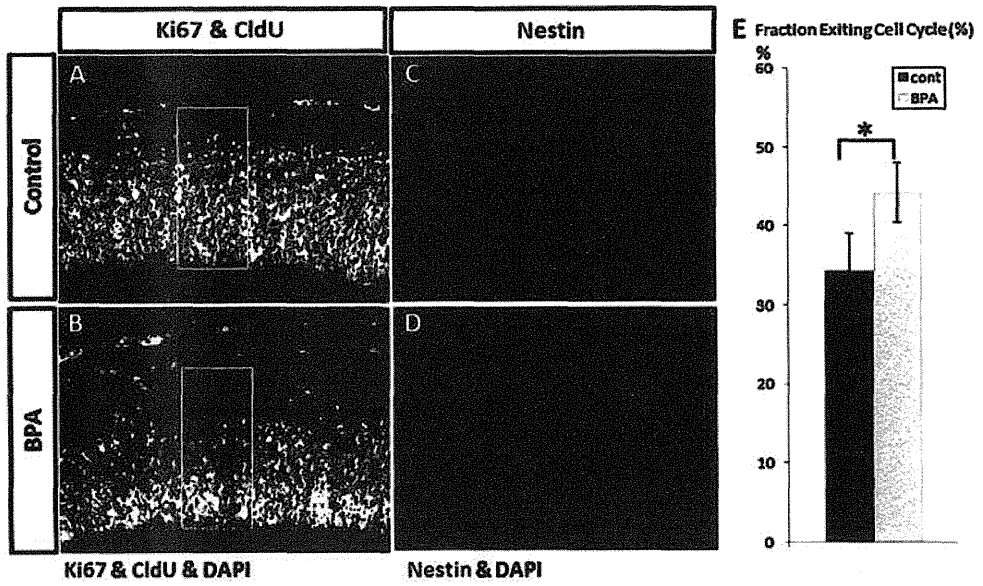

Fig. 2. The maternal oral exposure to BPA leads to acceleration of cell cycle exit and decreases the number of neural stem/progenitor cells. To estimate cell cycle exit of neural stem/progenitor cells, sections were stained with anti-Ki67 and anti-CldU antibodies 24 h after CldU pulse labeling at E14.5 in the control (A) and BPA-treated (B) fetuses. Parasagittal sections of E14.5 dorsal telencephalon were immunostained with anti-Nestin antibody. In BPA-treated fetuses, Nestin-stained radial fibers were shorter than those of the controls (C, D). (E) Cell cycle exit was determined as ratio of cells that exited the cell cycle (red, CldU+/Ki67−, no longer dividing) to all cells labeled with CldU (red + yellow) after 24 h labeling. Cells were counted in 100-μm-wide sampling boxes (white boxes in A and B). In the BPA-treated fetuses, the ratio was significantly increased at E14.5 (control fetuses: 34.3 ± 4.7%, $n = 9$, BPA-treated fetuses: 44.1 ± 3.8%, $n = 9$, $^*P < 0.01$). Scale bar: 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and the basal part of the SVZ/VZ in synchrony with the cell cycle. Thus, we performed immunostaining using anti-Nestin (the marker of radial fibers of RGCs) antibody in the parasagittal sections. In the BPA-treated fetuses, the Nestin-positive radial fibers were shorter than control ones at E14.5 (Fig. 2C and D). These data suggested that the short radial fibers resulting from the abnormal interkinetic nuclear migration in the SVZ/VZ and abnormal positioning of Ki67+ cells and double-positive (Ki67+ and CldU+) cells.

3.3. Reduction of proliferative neural stem/progenitor cells in the dorsal telencephalon

Accelerated neurogenesis might impact on the proliferation of neural stem/progenitor cells in the dorsal telencephalon. To examine whether cell proliferation was affected in the BPA-treated fetuses, we performed immunostaining using anti-Ki67 antibodies (Fig. 3A and B). At E14.5, the Ki67-immunopositive cell index (Ki67-positive cells/DAPI-stained cells) was significantly decreased in the dorsal telencephalon of BPA-treated fetuses (41.6 ± 1.0%, $P < 0.01$) compared with that in the control fetuses (47.2 ± 1.0%) (Fig. 3C). These data suggested that the maternal BPA oral dosing related to the reduction of neural stem/progenitor cells as a result of the promotion of neurogenesis in the dorsal telencephalon.

3.4. BPA specifically affected the intermediate progenitor cells

During the corticogenesis of neocortex, three different types of neural stem/progenitor cells (RGCs, IPCs, and outer radial glial cells) exist in the SVZ/VZ of the dorsal telencephalon (Molnar et al., 2011). These neural stem/progenitor cells are distinguished by their distribution, self-renewal ability, capacity for neurogenesis and expression of transcriptional factors (Molnar et al., 2011). To determine which cell population (RGCs or IPCs) was affected by BPA exposure, we performed immunostaining using anti-Pax6 (transcriptional factor, the marker of RGCs) and anti-Tbr2 (transcriptional factor, the marker of IPCs) (Fig. 4A–D). Pax6-positive cells represent RGCs in VZ and Tbr2-positive cells are IPCs in SVZ. There was no significant difference in the number of Pax6-positive cells between the BPA-treated (54.1 ± 3.1%) and control fetuses (55.1 ± 3.8%) (Fig. 4E). However, the number of Tbr2-positive cells was significantly decreased in the BPA-treated fetuses

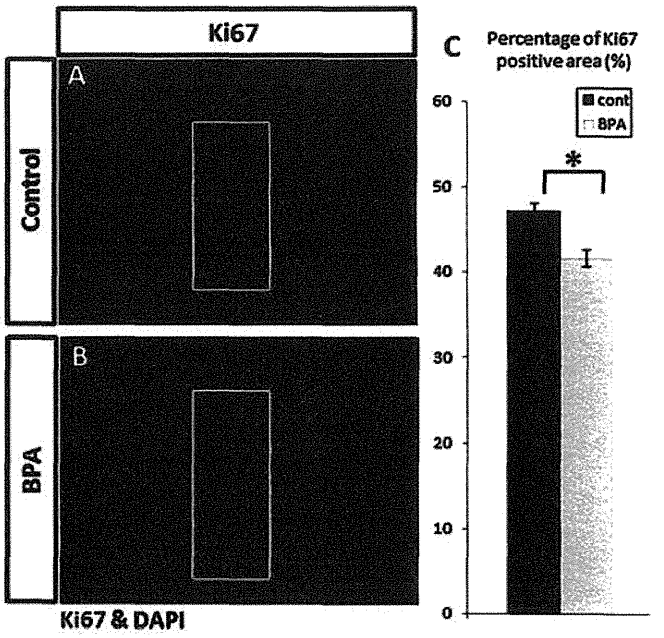

Fig. 3. Proliferation of stem/progenitor cells in the dorsal telencephalon is reduced by maternal BPA exposure. Immunostaining with Ki67 (red) of sagittal sections of E14.5 (A, B) fetal brains treated with BPA. Ki67 was expressed in the proliferative cells in the SVZ/VZ of dorsal telencephalon. Cells were counted in 100-μm-wide sampling boxes (white box). Examples of anti-Ki67 labeling of the dorsal telencephalon of the control (A) or BPA-treated fetuses (B). C: BPA-treated fetuses showed significant decrease in the ratio of Ki67+ cells at E14.5 (control fetuses: 47.2 ± 1.0%, $n = 9$, BPA-treated fetuses: 41.6 ± 1.0%, $n = 9$, $^*P < 0.01$). Scale bar: 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

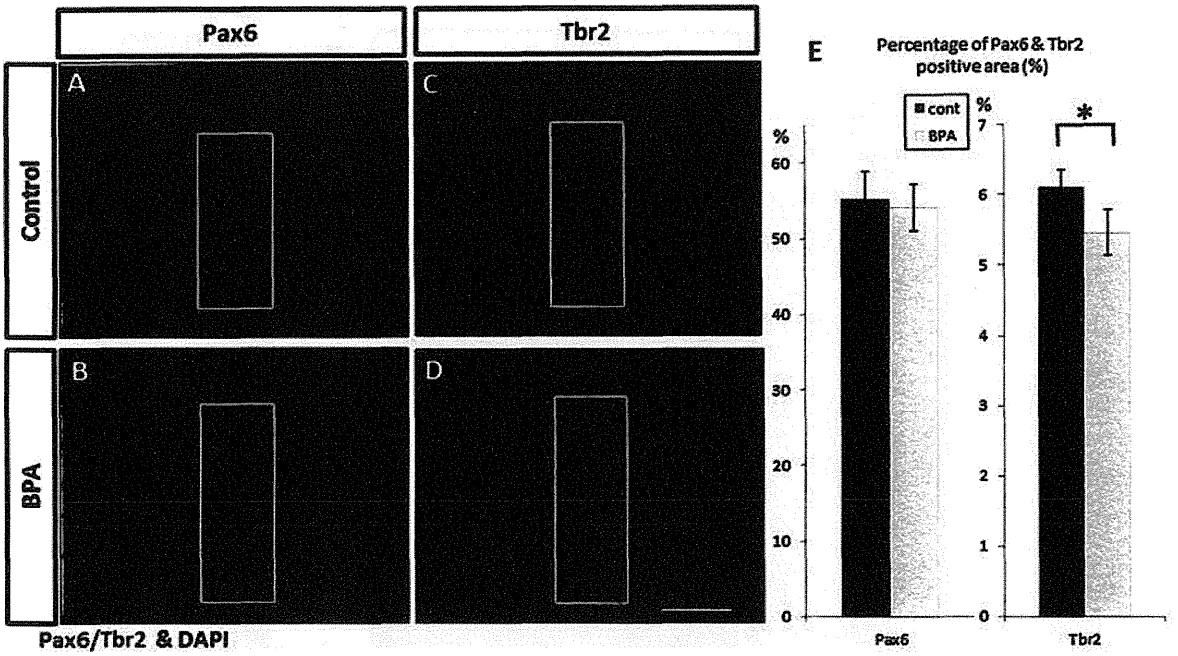

Fig. 4. Maternal BPA oral dosing induces the reduction of intermediate progenitor cells and does not affect radial glial cells. Immunostaining on coronal sections of E14.5 dorsal telencephalon with anti-Pax6 (A, B) and anti-Tbr2 (C, D). The Pax6-positive cells were considered as RGCs in VZ of the control (A) and BPA-treated fetuses (B) at E14.5. Cells were counted in 100-μm-wide sampling boxes (white box). There was no significant difference in Pax6-positive RGC number between the control and BPA-treated groups (E: control fetuses: 55.1 ± 3.8%, $n = 9$, BPA-treated fetuses: 54.1 ± 3.1%, $n = 9$). The Tbr2-positive cells were identified as intermediate progenitor cells (IPCs) in SVZ of the control (C) and the BPA-treated fetuses (D). (E) Tbr2-positive IPCs of the BPA-treated fetuses (6.12 ± 0.24%, $n = 9$, $^*P < 0.05$) showed significant reduction compared with those in the control fetuses (5.46 ± 0.32%, $n = 9$). Scale bar, 100 μm.

(5.46 ± 0.32%, $P < 0.05$) compared with that in the control fetuses (6.12 ± 0.24%) (Fig. 4E). These data suggested that the maternal BPA oral dosing specifically associated with the maintenance of IPCs in the SVZ of dorsal telencephalon.

3.5. The acceleration of RGCs and IPCs differentiation by BPA exposure

During the corticogenesis of neocortex, RGCs differentiate to IPCs or neurons and IPCs differentiate to neurons. IPCs mainly produce the projection neurons in layer II/III of neocortex. In evolution, layer II/III is among the most highly evolved regions and IPCs might have important roles in the progression of neocortex. Thus, to determine which cell (RGCs or IPCs) differentiation was affected by maternal BPA exposure, we performed cell cycle exit analysis using thymidine analog labeling with each cell. RGCs and IPCs are proliferative cells labeled by CldU in the SVZ/VZ. In the CP and upper SVZ after 24 h of CldU injection, CldU positive and Pax6/Tbr2 negative cells are defined as postomitotic (differentiated) cells from RGCs/IPCs, respectively. Differentiated RGCs and IPCs were identified by double-immunostaining using anti-Pax6/anti-CldU and anti-Tbr2/anti-CldU antibodies, respectively (Fig. 5A–D). The quantification of these experiments showed that the cell cycle exits of RGCs (37.4 ± 2.4%, $P < 0.01$) and IPCs (35.7 ± 1.2%, $P < 0.01$) in the BPA-exposed fetuses were significantly promoted compared with those in the control fetuses (RGCs: 32.8 ± 2.8%, IPC: 30.7 ± 3.7%) at E14.5 (Fig. 5E). These results indicated that the maternal BPA treatment related to the acceleration of neurogenesis of IPCs in the SVZ and differentiation of RGCs in the VZ of dorsal telencephalon.

3.6. BPA leads to prolongation of cell cycle of IPC

To investigate the cause of reduction of IPCs (Fig. 4), we examined the cell cycle length of IPCs. We hypothesized that prolongation of the cell cycle results in decreased proliferation of IPCs in the SVZ of dorsal telencephalon. We performed double immunostaining using anti-Tbr2 and anti-IdU antibodies (Fig. 6A and B). Cell cycle length of IPC was calculated as the rate of the total number of IPCs (Tbr2-positive cell) and the number of S-phase IPCs for 1 h (Tbr2/IdU double-positive cell). A smaller population of IdU-labeled cells among Tbr2-positive cells indicates a greater cell cycle length (Chenn and Walsh, 2002). In the BPA-treated fetuses, the cell cycle was significantly prolonged (8.4 ± 0.6%, $P < 0.01$) compared with that in the control fetuses (16.5 ± 4.3%) at E14.5 (Fig. 6C). These data indicated that the extension of the cell cycle length of IPCs induced the reduction of self-renewal, resulting in the decreasing number of IPCs.

4. Discussion

In previous studies, exposure to BPA (20 μg/kg/day) induced the accelerated neuronal differentiation/migration in ICR mice (Nakamura et al., 2006). Our data indicated that treatment with BPA at the dose of 20 μg/kg/day showed no differences in the morphogenesis of CP (Sup. Fig. 1A–C') and neurogenesis of neural stem/precursor cells (Sup. Fig. 1D–I, Sup. Table 2) at E14.5 between the BPA-treated and control fetuses in C57BL/6J mice (see the materials and methods). We hypothesized that the difference of phenotype resulted from the different administration routes (intraperitoneally or orally) or mouse strains used, with variation in sensitivity to BPA. In a previous study involving the maternal BPA oral dosing, number of fetuses born per litter and embryonic body weight were significantly lower in the group exposed to BPA at 200 μg/kg/day than those in the control group. However, no difference was detected between the group exposed to BPA at 20 μg/kg/day and the control group (Cagen et al., 1999). Our data also showed that histological changes (cell cycle, proliferation, and differentiation changes) were related to BPA at 200 μg/kg/day. However, these were not found after oral exposure to BPA at 20 μg/kg/day (Sup. Fig. 1, Sup. Table 2).

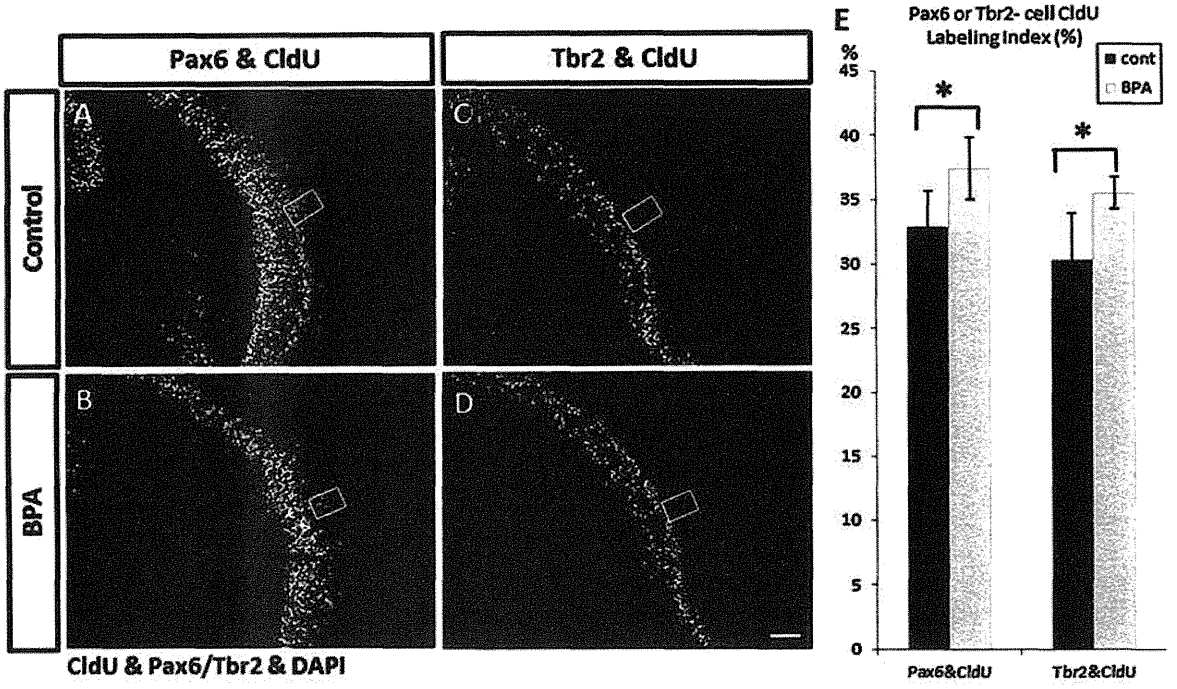

Fig. 5. Differentiation of radial glial cells and intermediate progenitor cells is promoted by maternal BPA exposure. To estimate the differentiation of RGCs and IPCs, sagittal sections were stained with anti-Pax6 or -Tbr2 and anti-CldU antibodies 24 h after CldU pulse labeling at E14.5 in the control (A, C) and the BPA-treated (B, D) fetuses. Differentiation was determined as the ratio of cells that differentiated from RGCs (green, CldU+/Pax6−, A, B) and IPCs (green, CldU+/Tbr2−, C, D) to all cells (blue: DAPI) after 24 h labeling. Cells were counted in 100-μm-wide sampling boxes (white box) in the SVZ (RGC, A, B) or upper SVZ (IPC, C, D). (E) In the BPA-treated fetuses, the ratio of differentiated cells from RGCs was significantly increased at E14.5 (control fetuses: 32.8 ± 2.8%, $n = 9$, BPA-treated fetuses: 37.4 ± 2.4%, $n = 9$, $^*P < 0.01$) and the ratio of differentiated cells from IPCs was also significantly increased at E14.5 (control fetuses: 30.3 ± 3.7%, $n = 9$, BPA-treated fetuses: 35.7 ± 1.2%, $n = 9$, $^*P < 0.01$). Scale bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

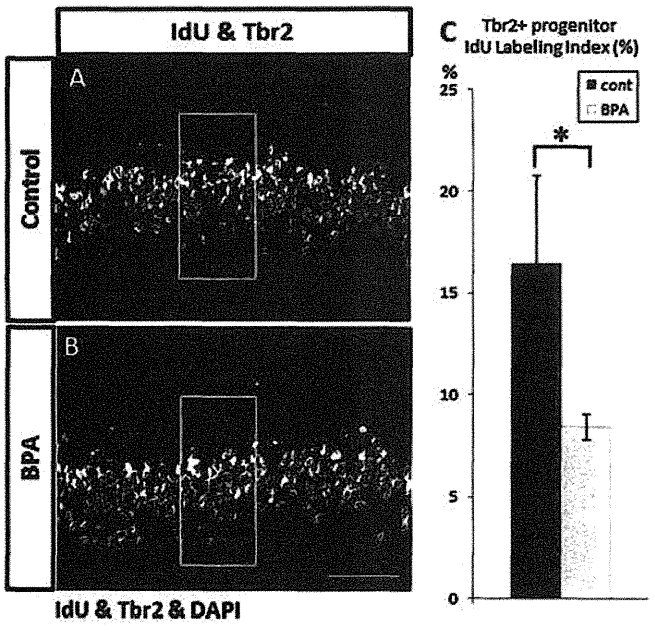

Fig. 6. The maternal exposure to BPA leads to prolonged cell cycle of intermediate progenitor cells. After 1 h pulse labeling of IdU, immunostaining of coronal sections was performed with anti-Tbr2 (red) and anti-IdU (green) antibodies at E14.5 (A, B). Cell cycle length was estimated as percentage of Tbr2 and IdU double-positive cells among all Tbr2-positive cells. Smaller percentage represents longer cell cycle. Cells were counted in 100-μm-wide sampling boxes (white box). (C) The BPA-treated fetuses showed significantly prolonged cycle length of IPC (8.4 ± 0.6%, $n = 9$, $^*P < 0.01$) compared with control fetuses (16.5 ± 4.3%, $n = 9$) at E14.5. Scale bar: 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

BPA, an endocrine disruptor, induces the promotion of neurogenesis and regulation of proliferation in neural stem/precursor cells. BPA is known as an estrogenic chemical that interacts with human estrogen receptor (ER) (Krishnan et al., 1993), acts as an antagonist for human androgen receptor (Xu et al., 2005) and strongly binds to human estrogen-related receptor gamma (Takayanagi et al., 2006). ERs and AR are expressed in many areas of the developing brain in rodents (Matsuda et al., 2008; Zsarnovszky and Belcher, 2001). In the development of CNS, estrogens have neurotrophic and differentiation-promoting effects. ERα and ERβ are crucial for the functions during a critical period of brain development (Beyer, 1999). In analysis using ERβ knockout mice, ERβ was shown to play important roles in the neuronal migration, differentiation and survival in the developing brain (Wang et al., 2003). In addition, BPA increases ERα expression in the brain (Bromer et al., 2010). BPA may also enhance estrogenic activity through the up-regulation of ERs and up- or down-regulation of estrogenic target gene expression in the target tissues. Presently, we are examining whether the estrogen and/or ER are related to these phenotypes and attempting to identify the impaired expression of estrogenic target genes after BPA exposure using our BPA exposure model fetuses.

In addition, BPA binds to protein disulfide isomerase (PDI), the same binding site as thyroid hormone 3,3′,5-triodo-L-thyronine (T3) (Hiroi et al., 2006). Thyroid hormone is essential for proper brain development. In the neocortex, thyroid hormone deficiency during development results in abnormal cytoarchitecture and neuronal network formation (Cuevas et al., 2005). Thyroid hormone receptor alpha and beta were expressed in the cerebral cortex and hippocampus of rat brain (Di Liegro, 2008). The results of the present study suggest that BPA has possibilities of interaction with each factor (receptors and/or enzymes) during the development of neocortex and can induce excessive signaling of these factors.

Our data suggested that BPA exposure inhibited the proliferation and promoted the differentiation of RGC (Pax6-positive cells) and IPC (Tbr2-positive cells) in the SVZ/VZ of dorsal telencephalon. ERβ is detected in the deep layer of the dorsal telencephalon at E16.5 (Fan et al., 2006), indicating that RGC and/or IPC in the SVZ/VZ expressed the ERβ. In addition, the estrogen signaling might maintain the stem cell niche of RGC and IPC and regulate neurogenesis (Wang et al., 2003). We hypothesized that BPA acts as an agonist of ER and induces excessive estrogen signaling in the dorsal telencephalon, resulting in the promotion of neurogenesis.

A recent study based on cellular behavior, morphology and gene expression pattern identified three similar progenitors during the development of neocortex: outer radial glial cells, RGCs and IPCs. RGCs span the width from ventricular to pial surface, performing self-renewal from symmetric divisions and producing neurons from asymmetric divisions (Molnar et al., 2011). IPCs are derived from asymmetric divisions of RGCs. IPCs express the transcriptional factor Tbr2 and divide within the SVZ (Noctor et al., 2004). IPCs mainly produce projection neurons in layer II/III of the neocortex during the later neurogenesis and are very important for the evolutional process of the human brain, upgrading and becoming enormous in the neocortex. In the BPA-treated model, our data indicated that the abnormality of IPC resulted in neurogenesis defect and aberrant corticogenesis. Accordingly, it is reasonable to suggest that the detection of IPC defects is essential for the risk assessment of chemical exposure in the development of human brain.

BPA exposure induced the alteration of gene expression associated with neurogenesis. Basic helix-loop-helix (bHLH) genes have two types, repressor type and activator type. Repressor types, such as Hes1 and Hes5, maintain the neural stem cells and promote gliogenesis; activator types, Mash1, Mash2 and Ngn2, accelerate neurogenesis. Nakamura et al. reported that Mash1 and Ngn2 were significantly up-regulated in BPA-treated embryos at E14.5 (Nakamura et al., 2006). In addition, we also indicated that Mash1 was up-regulated at E14.5 in BPA-treated embryos (data not shown). These data suggested that BPA affected the expression of activator-type bHLH gene, Mash1 or Ngn2, causing accelerated neurogenesis.

5. Conclusion

In our studies, the maternal BPA oral dosing related to hyperplasia of CP during the development of telencephalon (Fig. 1) and shortened the radial fibers of RGCs in the SVZ/VZ (Fig. 2). The phenotypes were induced by the accelerated neurogenesis of neural stem/progenitor cells in the dorsal telencephalon (Fig. 2). In addition, BPA associated with the reduction of neural stem/progenitor cells in the SVZ/VZ as a result of the promotion of neurogenesis in the dorsal telencephalon (Fig. 3). In particular, BPA related to the maintenance and neurogenesis of IPCs in the SVZ of dorsal telencephalon (Figs. 4 and 5). These phenotypes (accelerated neurogenesis and the reduction of IPC number) were induced by the extension of the cell cycle length of IPCs in the SVZ (Fig. 6). In the near future, we will clarify whether these morphological defects in the embryonic brain persists, and result in the functional aberrations after birth. The maternal BPA dosing associated with the disruption of cell cycle in IPCs and related to the effects on neurogenesis in the development of neocortex of fetuses.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2012.02.013.

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Review

Transgenerational Teratogenesis by Prenatal Exposure to Endocrine Disrupting Chemicals[1]

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Congenital malformations can be induced in the offspring of laboratory animals treated with the mutagens, ethylnitrosourea, methylnitrosourea, mitomycin C, triethylenemelamine or procarbazine before copulation. The spectra of malformations in the offspring classified as male-mediated malformations after exposure of paternal mice to mutagens showed no evidence of mutagen-specificity or germ-cell stage-dependent variations. Recently, we demonstrated the increased incidence of congenital malformations in the offspring of male mice exposed *in utero* to synthetic estrogens such as diethylstilbestrol (DES), 17β-estradiol (E_2) or ethynyl estradiol (EE), and that the induction of male-mediated malformations by DES, E_2 or EE showed a clear threshold effect. Developmental exposure to DES, E_2 or EE caused partial atrophy and feminization in the genital tract. They also showed transgenerational effects when applied prenatally at a dose which caused histopathological changes in the testes. Germ-cell series in normal testis have mechanisms to select against spontaneously arising mutation; but these selection mechanisms may not function efficiently in chemically-damaged testes. Based on these results and considerations, we propose as a hypothesis that transgenerational teratogenesis by prenatal exposure to synthetic estrogens may occur as a consequence of testicular toxicity. Moreover, since DES has been reported to be non-genotoxic, epigenetic mechanisms such as DNA methylation may be involved in the transgenerational teratogenesis induced by estrogenic drugs. The expression patterns of DNA methyltransferases (Dnmts) mRNA, global DNA methylation levels in testicular cells of embryos exposed to estrogenic drugs or in epididymal sperm of mature male mice exposed prenatally to estrogenic drugs were different from those in the controls. Results shown in this review support the proposal that, when evaluating the toxicities of environmental chemicals including endocrine disruptors, epigenetic effects such as DNA methylation should be taken into account.

Key words: transgenerational effects, congenital malformations, male-mediated teratogenesis, endocrine disruptor, synthetic estrogens, epigenetics, developmental exposure

Introduction

Although induction of germinal mutations by chemicals is well documented in experimental animals, there is no firm evidence that any agent has induced germinal mutations in men. Direct study of chemically-induced transmitted genetic effects in humans is virtually impossible, so the genetic risk must be estimated from animal experiments. Congenital malformations can be induced in the offspring of laboratory animals exposed to environmental mutagens before copulation (1–6). The spectra of congenital malformations in the offspring classified as male-mediated malformations after exposure of paternal mice to mutagens showed no evidence of mutagen-specificity or a germ-cell stage-dependent variation. More importantly, none of the spectra differed significantly from the type distribution of spontaneous malformations. In addition, when a teratogen was applied at the organogenic stage, embryos whose sires were preconceptually exposed to a mutagen suffered malformations with a higher frequency than those derived from untreated males. Thus, paternal exposure to mutagens can enhance susceptibility of F_1 embryos not only to "spontaneous teratogenesis", but also "induced-malformations" (7).

Estrogenic drugs may have transgenerational toxicity. Exploration of this possibility in animal models is justified due to the increasing concern about the health effects of endocrine disruptors in the environment. However, little is still known about such effects, except for the potential of a transgenerational carcinogenic effect of diethylstilbestrol (DES) reported in mice by Walker (8) and others. DES has been shown to have reproductive tract teratogenicity and carcinogenicity in both women and laboratory animals, and a practical concern was whether children born to women prenatally exposed to DES would have an increased risk of cancer

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(8). The studies carried out using CD-1 mice by Walker (8), Walker and Kurth (9) and Newbold *et al.* (10) showed that prenatal or neonatal exposure of females to DES can increase the incidence of reproductive tract tumors in female offspring. In a study carried out by Walker and Kurth (9) using the embryo-transfer technique, both maternal and germ cell-related pathways were identified for their transmission of carcinogenic risk of DES through females. These results imply that estrogenic drugs may cause persistent modification of germ cells when applied developmentally, and therefore they may exert a transgenerational effect.

We have demonstrated a significant increase in the incidence of congenital malformations in the offspring of male mice prenatally treated with DES, ethynyl estradiol (EE) or 17β-estradiol (E_2) (11). In our study, male mice were transplacentally exposed to estrogenic drugs on days 9 through 16 of gestation, and their fetal offspring were examined for evidence of transgenerational teratogenic effects due to the drugs. The teratogenic effect on external characteristics was examined because it is expressed early in life and can be monitored easily either phenotypically or quantitatively. Outbred ICR mice were used for the treatment and as untreated mating partners for the treated males because teratogenesis mediated by mutagenized germ cells has been studied extensively using this strain. With the treatment schedule used in our study, primordial germ cells (PGCs) were exposed to estrogenic drugs. Critical evidence for the susceptibility of mouse PGCs to genetic damage was reported by Shibuya and co-workers (12,13). They treated transplacentally C3H/He mice with varying doses of ethylnitrosourea (ENU) on gestational day 8.5, 10.5 or 13.5 and examined the incidence of recessive visible mutations at six specific-loci in F_1 of a cross of the treated males × tester females from the PW strain. The results led them to conclude that mutations at specific-loci can be induced in the PGCs by ENU at a several fold higher rate than in stem cell spermatogonia. Vulnerability of PGCs to ENU was further confirmed by Wada *et al.* (14), who examined skeletal malformations in the offspring of male mice transplacentally treated with this agent on gestational day 10.5. In this review, we show the characteristics of male-mediated teratogenesis of environmental mutagens, and demonstrate that the treatment of male mice at the PGC stage with estrogenic drugs caused a clear increase in the incidence of congenital malformations in the subsequent generation.

Congenital Malformations in the Offspring of Male Mice Exposed to Potent Mutagens

Transgenerational teratogenesis experiments (male-mediated teratogenesis): In the experiments on male-mediated congenital malformations, male mice were exposed to mutagens. The treated males were individually caged with untreated virgin females of the same strain (15). The mating intervals were days 1–21 or days 64–80 after the last dosing. Copulations during these periods involved, respectively, treated post-meiotic cells (spermatozoa and spermatids) and pre-meiotic cells (spermatogonial stem cells) at the time of treatment. Presence of a vaginal plug defined day 0 of pregnancy and pregnant females copulated with treated males were killed on day 18 of gestation to observe the fetal morphology. Fetal malformations inspected were gross external and skeletal abnormalities. Significantly enhanced frequency of malformed fetuses per live fetus in the treated series over the control level was taken as evidence of male-mediated teratogenesis, and the abnormalities detected were collectively referred to as F_1 malformations.

Germ cell stages at risk for male-mediated teratogenesis: Male germ cells from primordial to post-meiotic stages are continuously at risk of the induction of F_1 malformations by exogenously applied agents, as evidenced by the data shown in Table 1 for urethane (15,16) and ENU (2,14). It is also clear from Table 1 that all the agents that were effective in inducing F_1 malformations are known mutagens in mice and other *in vivo* systems. Taken together, there is no doubt that genetic damage to the male-germ-line is the mechanism of male-mediated teratogenesis induced by exposure to environmental mutagens.

Possible nature of germ-line mutations responsible for transgenerational teratogenesis induced by mutagens: With a specific-locus test for seven visible markers, six compounds were identified as effective inducers of heritable point mutations in spermatogonial stem cells; i.e., triethylenemelamine (TEM) (23), mitomycin C (MMC) (24), ENU (25), methylnitrosourea (MNU) (26), procarbazine (PCZ) (27), propyl methanesulfonate (PMS) (28). As shown in Fig. 1, all these agents were effective in producing male-mediated congenital malformations when applied at the spermatogonial stem cell stage in ICR mice (4). From the dose-response curves shown in Fig. 1, we estimated the genotoxically effective dose FD_2 in mmole/kg, the dose required to produce externally malformed fetuses with a frequency of 2%, to be 0.07 for TEM and MMC, 0.6 for ENU and MNU, 1.8 for PCZ, and 3.0 for PMS (Table 2). These dose values demonstrate that TEM and MMC are the strongest mutagens for producing male-mediated malformations, followed by (ENU, MNU), PCZ and PMS in that order. From dose-response data on specific-locus mutations, we estimated $MD_{0.02}$ in mmole/kg, the dose required to produce visible mutations at the seven loci with an average frequency of 0.02%, to be 0.006 for TEM, 0.01 for MMC, 0.3 for ENU, 0.2 for MNU, 1.3 for PCZ and 4.6 for PMS.

Table 1. Mutagens tested for induction of F_1 malformations in mouse and rat

Mutagen	Germ cell stage (Species)	Effect	Reference
TEM	SG (Mouse)	+	4
MMC	SG (Mouse)	+	4
ENU	PGC (Mouse)	+	14
	PM (Mouse)	+	2,17
	SG (Mouse)	+	2,17
MNU	PM (Mouse)	+	1
	SG (Mouse)	+	1
PCZ	SG (Mouse)	+	4
PMS	SG (Mouse)	+	4
X-rays	PM (Mouse)	+	15,17,18
	SG (Mouse)	+	15,17,18
Urethane	PGC (Mouse)	+	16
	PM (Mouse)	+	15,17
	SG (Mouse)	+	15,17
CPA	PM (Mouse)	+	19
EMS	PM (Mouse)	+	20
DMBA	PM (Mouse)	+	17
	SG (Mouse)	−	17
AA	SG (Mouse)	−	Nagao (unpublished)
AF-2	PM (Mouse)	−	17
4NQO	PM (Mouse)	−	17
EGM	SG (Mouse, Rat)	−	21
TCDD	SG (Rat)	−	22

+, positive; −, negative

Abbreviations used for mutagens: TEM, triethylenemelamine; MMC, mitomycin C; ENU, ethylnitrosourea; MNU, methylnitrosourea; PCZ, procarbazine; PMS, propyl methanesulfonate; CPA, cyclophosphamide; EMS, ethyl methanesulfonate; DMBA, 7,12-dimethylbenz (a) anthracene; AA, acrylamide; AF-2, 2-(furyl)-3-(5-nitro-2-furyl)-acrylic acid amide (furylfuramide); 4NQO, 4-nitroquinoline l-oxide; EGM,ethylene glycol monomethyl ether; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Abbreviations used for germ cell stage: SG, spermatogonial stem cells; PGC, primordial germ cells; PM: post-meiotic germ cells.

Again, the ranking of the genotoxic potency was TEM = MMC > ENU = MNU > PCZ > PMS. Furthermore, the FD_2 value was very close to the $MD_{0.02}$ value for all mutagens used. We thus concluded that, in mutagenized spermatogonial stem cells, genetic changes responsible for male-mediated malformations are produced in a manner similar to that involved in the production of specific-locus mutations. This means that they probably represent point mutations, namely, genetic changes not associated with gross rearrangements.

The excellent correlation between FD_2 and $MD_{0.02}$ further implies that the number of target loci per genome for the production for male-mediated F_1 malformations is two orders of magnitude higher (i.e., 2/0.02) than that for the specific-locus mutations (4). A similar conclusion was derived from the X-ray results of studies by Nomura (17).

A high percentage of malformations in fetuses from mutagenized paternal germ cells are the result of increased yields of spontaneously occurring mal-

Table 2. Genotoxic potency of the 5 mutagens to induce F_1 fetal abnormalities and specific-locus mutations in spermatogonial stem cells

Mutagen	F_1 fetal abnormalities	Specific-locus mutations
	FD_2^* (mmole/kg)	$MD_{0.02}^{\dagger}$ (mmole/kg)
TEM	0.007	0.006
MMC	0.007	0.01
ENU	0.6	0.3
PCZ	1.8	1.3
PMS	3.0	4.6

*Effective dose for producing F_1 fetal abnormalities with a frequency of 2%. Data are from references (2,4).

†Effective dose for producing specific-locus mutations with frequency of 0.02%. Data are from references (23–25,27,28) See footnote of Table 1 for the abbreviations used for mutagens.

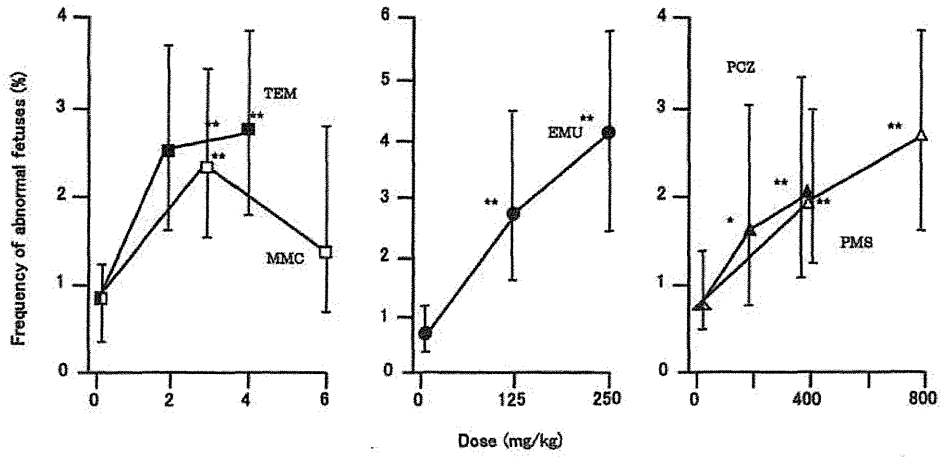

Fig. 1. Dose-response relations of malformed fetuses observed after exposure of ICR mice to chemical mutagens at spermatogonial stem cell stage. Data are from references (2,4). The vertical lines represent 95% confidence intervals of the frequencies. See footnote of Table 1 for the abbreviations used for mutagens. *Significantly different from control, $p=0.05$. **Significantly different from control, $p=0.01$.

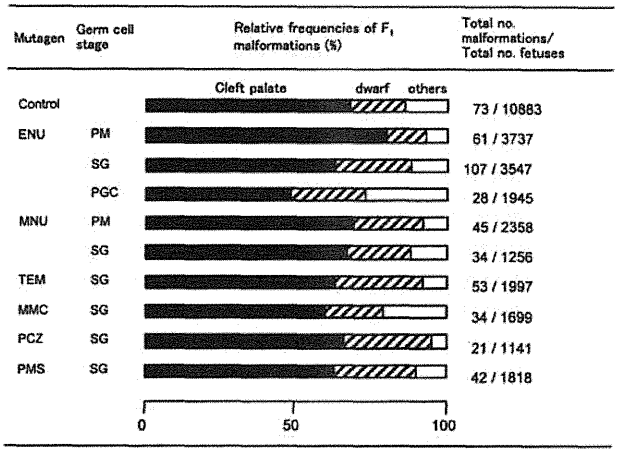

Fig. 2. Relative frequencies of external malformations detected in fetal offspring of ICR males exposed to various kind of mutagens at various germ-cell stages. Data are from references (1,2,4,14). See footnote of Table 1 for the abbreviations used for germ cell stages.

formations: Figure 2 shows the type-distribution of external malformations observed after treatment with various mutagens at the spermatogonial stem cell stage. In order to compare with type-distribution of spontaneously induced abnormalities, the data pooled for the concurrent and historical controls are also shown in Fig. 2. Among external malformations induced after treatment of spermatogonial stem cells, cleft palate was the most common, followed by dwarfism, and others (exencephaly and abnormal limbs such as polydactyly and syndactyly) in any mutagen-treated groups. Spectra of external abnormalities recorded as male-mediated congenital defects in any mutagen-treated groups were indistinguishable from each other, showing no evidence of a mutagen-specificity. More importantly, spectra determined after exposure of paternal germ cells to mutagens did not differ from the spectrum of spontaneously induced malformations. These results clearly characterize male-mediated teratogenesis, as the spectra of fetal malformations induced after treatment of embryos at the organogenic stage with these mutagens showed marked differences from the control spectrum. Among external abnormalities in embryos of ICR mice treated with ENU on day 8 of gestation, microphthalmia (27%) was the most common, followed by exencephaly (1.1%), cleft palate (0.8%) and hydrocephaly (0.8%) (3). Thus, we are inclined to hypothesize that the high percentage of external malformations in fetuses from mutagenized paternal germ cells is the result of increased yields of spontaneously occurring malformations. In other words, spontaneous fetal malformations may also arise, at least partly, as a genetic disease.

Figure 2 shows the type-distribution of external abnormalities detected in fetuses derived from post-meiotic germ cells and spermatogonial stem cells treated with ENU and MNU. In both mutagens, the main types of fetal abnormalities were cleft palate, dwarfism, exencephaly and abnormal limbs in treatments with both post-meiotic germ cells and spermatogonial stem cells. The spectra in different stages of paternal germ cells were indistinguishable, irrespective of the kind of mutagens used, showing no evidence of a germ-cell stage dependent variation. In summing up all the data on external malformations, it seems that the spectrum of external abnormalities in fetuses derived from mutagen-treated paternal germ cells mimics the spectrum of spontaneously occurring malformations and, as far as external abnormalities are concerned, evidence for male-mediated teratogenesis can be obtained only from a quantitative comparison of the incidence of malformed fetuses between the exposed and the control groups. Spontaneous spectrum of male-mediated malformations often showed a strain-dependent variation. The predominant types of external malformations were cleft palate and dwarfism in the ICR strain, microphthalmia and micrognathia in C57BL/6N (5), and cleft palate and open eyelids in A/Jax (6). In our studies with the ICR strain, the average frequency (cleft palate and dwarfism) was 2:1 in the ENU experiment. In the experiments reported by Kirk and Lyon (18), who used (C3H/HeHx101/H) F_1 hybrids, the ratio was 1:25. Thus, it is reasonable to suggest that the spectrum of induced male-mediated malformations also depends on the strain of mice used.

Modified susceptibility to induced-teratogenesis in the offspring of males exposed to environmental mutagens: Fetuses derived from males exposed to mutagens may also have enhanced susceptibility to induced-teratogenesis. "Induced-teratogenesis" means teratogenesis induced in the fetuses of mothers exposed to teratogens. This possibility was tested in an experiment where ICR females mated with ENU-exposed males of the same strain, and those mated with untreated males, were treated with ENU in the organogenic period, and the fetuses were inspected for external and skeletal malformations at term. As exemplified by the data in Fig. 3, there was a lot of evidence of increased sensitivity to microphthalmia as induced-malformations in the offspring of males exposed to mutagens. Thus, paternal exposure to mutagens can enhance susceptibility of F_1 embryos not only to spontaneous teratogenesis, but also to induced-malformations. Paternal exposure may increase the risk of fetal malformations due to the mother's exposure to drugs during pregnancy (7).

Transgenerational Adverse Events Induced by Endocrine Disruptors

The synthetic estrogen DES is a potent perinatal endocrine disruptor. In humans and experimental animals, exposure to DES during critical periods of

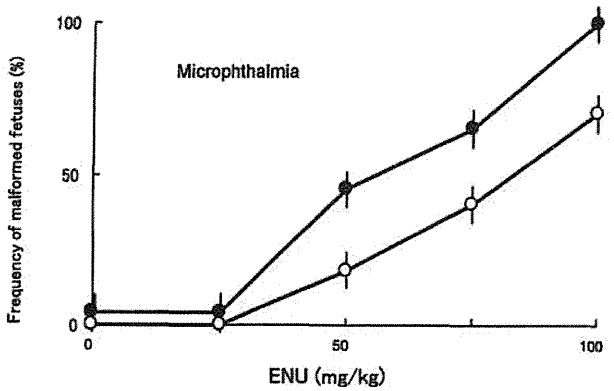

Fig. 3. Frequencies of fetuses with microphthalmia observed 10 days later after ENU exposure on day 8 of gestation to pregnant ICR females that had been conceived by ICR males exposed to ENU at spermatogonial stem cell stage (closed circles) or by untreated ICR males (open circles). Vertical lines represent standard errors of the frequencies. Data are from reference (7).

reproductive tract differentiation permanently alters estrogen target tissues and results in long-term abnormalities such as uterine neoplasia that are not manifested until later in life. Using mice exposed to DES developmentally, multiple mechanisms have been identified that play a role in its carcinogenic and toxic effects. Since mechanistic studies provided support that environmental estrogens cause both genetic and epigenetic alterations in developing target tissues (29–32), the possibility was raised that abnormalities seen following developmental exposure to DES could be transmitted to subsequent generations.

Exploration of this possibility in animal models is justified due to the increasing concern about the health effects of endocrine disruptors in the environment. However, little is still known about such effects, except for the potential for a transgenerational carcinogenic effect of DES reported in mice by Walker (8) and others. DES had been shown to have reproductive tract teratogenicity and carcinogenicity in both women and laboratory animals, and a practical concern was whether children born to women prenatally exposed to DES would have an increased risk of cancer (8). The studies carried out using CD-1 mice by Walker (8), and Walker and Kurth (9) showed that prenatal or neonatal exposure of female mice to DES can increase the incidence of reproductive tract tumors in female offspring. In a study carried out by Walker and Kurth (9) using the embryo-transfer technique, both maternal and germ-cell related pathways were identified for the transmission of carcinogenic risk of DES through females. Studies by Newbold *et al.* (10,33) showed that prenatal or neonatal exposure to DES led to tumors in the female and male genital tract, and in addition, the susceptibility for tumors was transmitted to the descendants through the maternal germ cell lineage. Mice were exposed to DES prenatally on days 9–16 of gestation, or neonatally on days 1–5. When female mice (F_1) reached sexual maturity, they were bred to untreated males. Female and male offspring (DES-lineage or F_2) from these matings were ages to 17–24 months and examined for genital tract abnormalities. An increased incidence of proliferative lesions of the rete testis, as well as tumors of the reproductive tract, was observed in DES-lineage males (33). Further, in DES-lineage females, an increased incidence of uterine adenocarcinoma was seen (10). These results suggest that this increased susceptibility to tumors is passed on from the maternal lineage to subsequent generations of male and female descendants; the mechanisms involved in these transgenerational events include genetic and epigenetic effects. Together, the data of Newbold *et al.* indicate the unique sensitivity of the developing organism to endocrine-disrupting chemicals, the occurrence of long-term effects following developmental exposure, and the possibility of adverse effects to be transmitted to subsequent generations (34). Multigenerational effects of DES have been reported by other laboratories and some of these reported transmission through the paternal lineage (35,36).

Increased incidence of congenital malformations in the offspring of male mice exposed to estrogenic drugs at the embryonic stage: We demonstrated the increased incidence of congenital malformations in the offspring of male mice exposed prenatally to a synthetic estrogen such as DES, EE, estradiol benzoate (EB), or E_2 (11,37). The results have shown that prenatal exposure of males to estrogenic compounds is hazardous not only for the development of the reproductive tract in the exposed mice, but also for embryonal development in the subsequent generation. In the groups showing male-mediated teratogenesis after transplacental exposure to an estrogenic compound on days 9 through 16 of gestation, anatomical or histopathological changes (unilateral atrophy, elongated configulation) were induced in testes, epididymides, or seminal vesicles of the males at the mature stage. Some males after prenatal exposure to estrogen at fairly high dose level had markedly dilated Müllerian duct remnants, which resembled the uteri. In the mature males of the group showing no male-mediated teratogenesis, no testicular damage was observed even by ultrastructural observation. Thus, it is reasonable to suggest that transgenerational teratogenesis by prenatal exposure to estrogenic compounds may occur as a consequence of their anatomical or histopathological testicular damages. As pointed out earlier, germ-cell series in normal testes have mechanisms to select against spontaneously arising mutations, but these selection mechanisms may not function efficiently in chemically-damaged testes (37).

In contrast to the reproductive tract abnormalities, all

of the external malformations detected in the fetal descendants of males exposed prenatally to estrogenic compounds were types known to occur in the ICR mice used in our studies. Namely, with regard to the types of malformations, the transgenerational effects observed in our studies were neither atypical for ICR mice nor specific to the drugs tested or the stage of the germ-cell development at the time of exposure. The relative ratios of cleft palate, dwarfism, and exencephaly in the groups exposed to EB-treated, EE-treated and control series were 1:0.3:0.07, 1:0.4:0.07 and 1:0.2:0.07, respectively. Similarly, cleft palate was the most common, followed by dwarfism and exencephaly in this order among external malformations produced in the offspring of ICR males treated with a mutagen at the spermatogonial stem cell or post-meiotic cell stage (1,2,4,7). Taken together, it seems that heritable damage was induced in embryonic germ cells (i.e., PGCs) after exposure to EB or EE, and the offspring inherited the damage and were sensitized to spontaneous teratogenesis. PGCs are at risk of chemical induction of transmissible changes, as shown by the induction of specific-locus mutations and dominant skeletal mutations with ENU (12,14). However, it is not clear whether the germ-cell damage responsible for the effects observed in our transgenerational teratogenic studies with synthetic estrogens was genetic or "epigenetic" in nature. Epigenetics is typically defined as the study of heritable changes in gene expression that are not due to changes in DNA sequences.

Of particular interest in our studies is that the induction of male-mediated malformations induced by the exposure to estrogenic drugs showed a clear threshold effect (11), and estrogenic drugs such as DES have been reported to be non-genotoxic (38), suggesting the non-genotoxic action of estrogenic drugs for transgenerational teratogenesis. Despite such uncertainty, the results of our studies on male-mediated teratogenesis of estrogenic drugs agree with those of previous reports on transgenerational carcinogenicity of DES (9,35) in suggesting vulnerability of developing germ cells to estrogenic drugs. Thus, the transgenerational effects of estrogenic drugs do not seem to be a rare phenomenon in mice. Studies of the molecular mechanisms of the effects are required to evaluate the implications of these observations in humans.

Epigenetic transgenerational inheritance of endocrine disruptors: A wide variety of chemical and physical agents have the potential to cause adverse effects by causing heritable changes to the genome, resulting in heritable alterations in phenotype. This is often thought, indeed assumed, to be a consequence of mutations, which may occur through either a genotoxic mechanism, or indirectly as a result of a non-genotoxic mechanism. A genotoxic mechanism involves either the agent itself or a metabolite of it interacting directly with DNA (39), thus, providing a substrate for mutagenesis. Alternatively, a non-genotoxic compound (i.e., the compound and its metabolites do not bind to DNA) may cause mutations through an indirect, secondary mechanism. For example, hyperplasia can occur in response to necrosis (40), and this may result in mutagenesis as a consequence of the less than perfect fidelity of DNA polymerase (41). However, mutagenesis is not the mechanism underlying heritable alterations to the genome (42). Indeed, it is appropriate to consider that heritable alterations in phenotype may also have an epigenetic basis (43–46).

Examples of probable epigenetic transgenerational effects are known. Mice treated with ionizing radiation or urethane produce offspring in which the frequency of tumors is greatly increased (15). It is very unlikely that this result could be due to the induction and transmission of new mutations in tumor suppressor genes. Instead, it may well be that epigenetic defects are being transmitted, which predispose cells to produce tumors (47). Another study has shown that irradiated mice produce offspring that are unusually sensitive to carcinogens, in comparison to animals with untreated parents (48). This again argues in favor of epigenetic transmission. There are other examples in the literature of such transmission (49).

Newbold *et al.* showed altered methylation patterns in several uterine genes that were permanently dysregulated following developmental DES treatment (50,51). The estrogen-responsive proteins lactoferrin (LF) and c-fos were permanently up-regulated in the uterus following developmental exposure to DES and the promoter regions of these genes were shown to be hypomethylated (50,51). Although the consequences of these types of alterations are unclear, studies suggested that methylation patterns can be passed to subsequent generations (52). A recent report by Skinner and his colleagues supports this theory since prenatal exposure to vinclozolin (an antiandrogenic compound) or methoxychlor (an estrogenic compound) caused adverse effects on testes morphology and male fertility, and that these effects were transmitted to subsequent generations (53). In addition, this report showed that these two chemical compounds caused epigenetic alterations in the DNA, specifically hyper- and hypo-methylation, and that these alterations were also observed in subsequent generations (53,54). Since the responses of estrogen regulated genes are set during development, altered hormone responses may be transmitted to subsequent generations.

In natural populations, both sexes may encounter affected, as well as unaffected, individuals during the breeding season, and any diminution in attractiveness could compromise reproductive success. Crews *et al.* (55) examined mate preference in male and female rats whose progenitors had been treated with vinclozolin.

This effect was sex-specific, and they demonstrated that females three generations removed from the exposure discriminate and prefer males who do not have a history of exposure, whereas similarly epigenetically imprinted males do not exhibit such a preference. These observations suggest that the consequences of endocrine disruptors are not just transgenerational, but can be transpopulational, because in many mammalian species, males are the dispersing sex. The results indicate that epigenetic transgenerational inheritance of endocrine disruptor action represents an unappreciated force in sexual selection (55).

The study of Dolinoy's group shows that maternal exposure to the endocrine disrupting chemical bisphenol A shifted the coat color distribution of viable yellow agouti (A^{vy}) mouse offspring toward yellow by decreasing CpG (cytosine-guanine dinucleotide) methylation in an intracisternal A particle retrotransposon upstream of the *Agouti* gene. DNA methylation at the A^{vy} locus was similar in tissues from the three germ layers, providing evidence that epigenetic patterning during early stem cell development is sensitive to bisphenol A exposure (56).

In women, prenatal exposure to DES is associated with adult reproductive dysfunction. The menstrual and reproductive characteristics in a unique cohort comprising daughters of women exposed prenatally to DES were reported (57). Their data provide evidence of menstrual irregularity and delayed menstrual regularization in the daughters of women exposed *in utero* to DES. The findings were the first documented example of transplacental carcinogenesis in humans, and are compatible with speculation regarding transgenerational transmission of DES-related epigenetic alterations in humans. Most of the outcomes seen in the women have been replicated in prenatally exposed mice (58–61); thus, the mouse model is useful for investigating DES-related mechanisms. Studies of developmentally exposed rodents indicate that DES exerts its influence on reproductive tissues through epigenetic mechanisms involving disrupted estrogen signaling and permanent changes in gene expression, probably due to altered DNA methylation (62–64). Rodent studies have identified altered expression in multiple genes, including those inducible by estrogen, such as LF, estrogen receptor, epidermal growth factor, and specific proto-oncogenes, as well as genes involved in the structural development of the reproductive tract, and those governing embryonic development (65–75).

Altered expression of DNA methyltransferases and genomic DNA methylation in testes of embryos exposed to estrogen in mice: As mentioned earlier, induction of male-mediated congenital malformations by estrogenic drugs in our studies showed a clear threshold effect. In addition, since DES has been reported to be non-genotoxic (38), epigenetic mechanisms such as DNA methylation may be involved in the transgenerational teratogenesis induced by estrogenic drugs.

DNA methylation is catalyzed by a family of DNA methyltransferases (Dnmts) that is composed mainly of Dnmt1, Dnmt3a, Dnmt3b and Dnmt3L. Dnmts are mainly involved in the maintenance of DNA methylation patterns following replication. Dnmt3a and Dnmt3b are involved in the establishment of *de novo* methylation (76). Expression patterns of Dnmts mRNA in the normal developing testes of mice have already been reported (77,78). The results of Sakai *et al.* (77,79) strongly suggest that it is not Dnmt1, but some other type of Dnmts that contributes to the creation of DNA methylation patterns in male germ cells, while Dnmt3a2 and Dnmt3L are responsible for the global DNA methylation in mouse male germ cells.

In our studies, C57BL/6N mice, an estrogen-sensitive strain (80), were treated with DES during pregnancy. Expression of Dnmt1, 3a, 3a2, 3b, and 3L mRNA in embryonic testes or epididymides of mature males were analyzed by real-time PCR. The expression patterns of Dnmts in testicular cells of embryos exposed to DES were different from those in controls (Fig. 4), suggesting that exposure of embryos to synthetic estrogens during the early developmental stage of gonads affects the Dnmts expression profile in germ cells. The alterations in the expression levels of Dnmts may be involved in the formation of aberrant DNA methylation. In addition, changes in global DNA methylation levels in the testes of embryos exposed to EE were found, as well as influence of the mRNA expression of imprinted genes *H19* and *Igf2* (insulin-like growth factor 2 gene) (Fig. 5) and altered genomic DNA methylation status of imprinted genes.

Recently, adverse effects induced by chemicals and current changes in DNA methylation have been reported in several laboratories. For example, exposure of mouse preimplantation embryos to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which binds to the aryl hydrocarbon receptor, reduced the fetal body weight and increased the methylation levels of the imprinted genes *H19* and *Igf2*, as well as Dnmt activity (81). Another report indicated that exposure of pregnant female rats during the developmental period of gonadal sex determination to vinclozolin or methoxychlor induced transgenerational adverse effects on male fertility and altered the DNA methylation patterns in the germline (53). In these cases, changes in DNA methylation may also be involved in the adverse processes that occur. Taken together, these results support the proposal that, when evaluating the toxicities of environmental chemicals including endocrine disruptors, epigenetic effects, such as DNA methylation, should be taken into account (42,82).

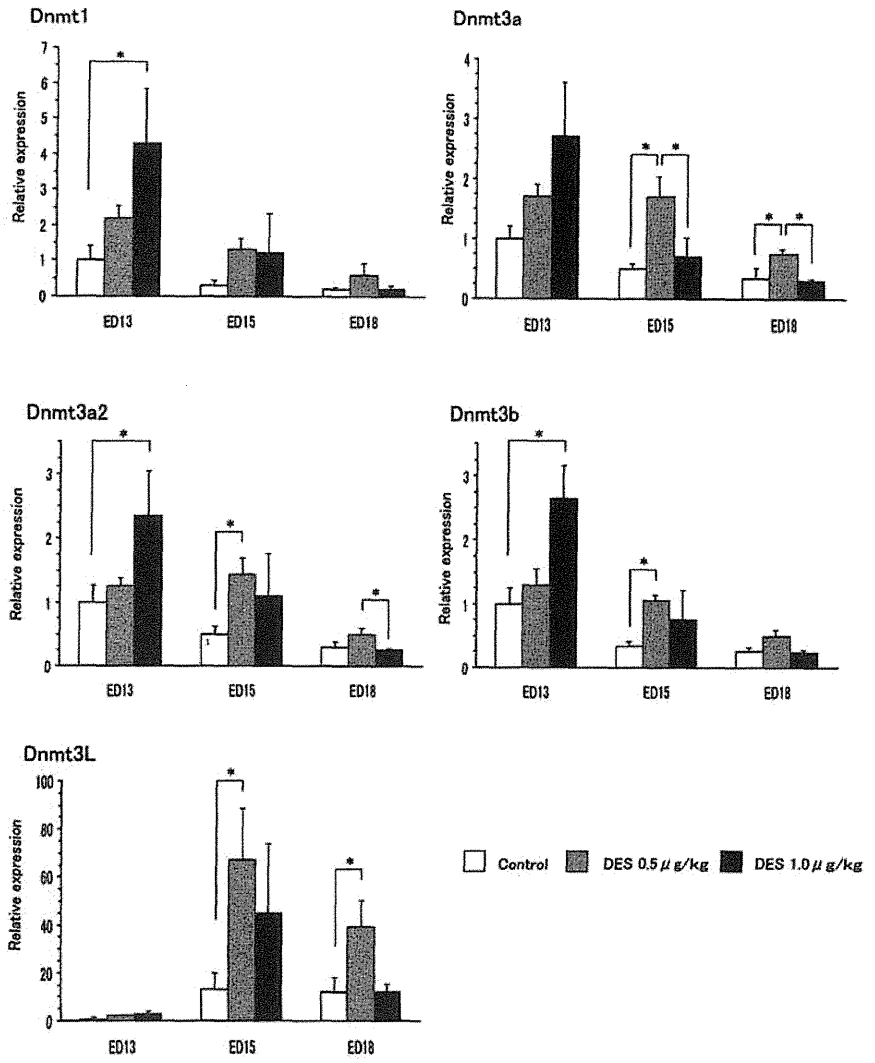

Fig. 4. Expression of Dnmts mRNA in the testes of ICR mouse embryos exposed to DES on days 8 through 11 of gestation. Real-time PCR was done. Vertical lines represent standard errors of the means. *Significantly different from control, $p=0.05$.

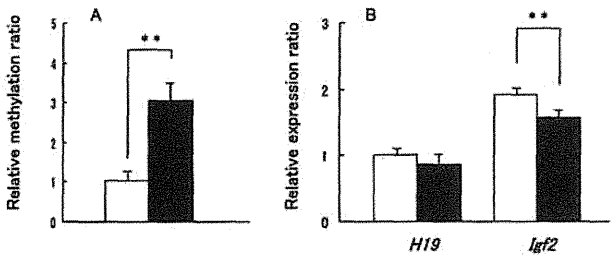

Fig. 5. Global DNA methylation (A) and real-time PCR analysis of mRNA expression of imprinted mouse *H19* and *Igf2* genes (B) in embryonic day 13 embryos exposed to EE. Expression ratios of targeted genes relative to the control. Open column and closed column represent control group and EE-exposed group, respectively. **Significantly different from control, $p=0.01$.

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Vesnarinone Suppresses TNFα mRNA Expression by Inhibiting Valosin-Containing Protein

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ABSTRACT

Vesnarinone is a synthetic quinolinone derivative used in the treatment of cardiac failure and cancer. It is also known to cause agranulocytosis as a side effect, which restricts its use, although the mechanism underlying agranulocytosis is not well understood. Here, we show that vesnarinone binds to valosin-containing protein (VCP), which interacts with polyubiquitinated proteins and is essential for the degradation of IκBα to activate nuclear factor (NF)κB. We show that vesnarinone impairs the degradation of IκBα, and that the impairment of the degradation of IκBα is the result of the inhibition of the interaction between VCP and the 26S proteasome by vesnarinone. These results suggest that vesnarinone suppresses NFκB activation by inhibiting the VCP-dependent degradation of polyubiquitinated IκBα, resulting in the suppression of tumor necrosis factor-α mRNA expression.

Introduction

Vesnarinone (3,4-dihydro-6-[4-(3,4-dimethosy-benzoyl)-1-piperazinyl]-2(1*H*)-quinolinone) is a quinolinone derivative developed as an inotropic agent for the treatment of congestive heart failure (CHF) (Cavusoglu et al., 1995) to modulate Ca^{2+} channels (Yatani et al., 1989). Vesnarinone is now known to have other activities, such as immunosuppressive activity (Matsui et al., 1994; Sato et al., 1995), the inhibition of human immunodeficiency virus production, the reduction of endotoxemic lethality, and the suppression of the growth of various tumor cell lines, including gastric cancer, lung cancer, adenoid squamous carcinoma, and myeloid leukemia (Fujiwara et al., 1997; Nio et al., 1997; Honma et al., 1999; Kubo et al., 1999; Yokozaki et al., 1999). However, the induction of agranulocytosis has been reported as a side effect of vesnarinone, thereby representing a limitation on its use (Cohn et al., 1998).

At the molecular level, vesnarinone appears to enhance myocardial contractility by augmenting sodium-calcium exchange (Yatani et al., 1989), which may be responsible for the treatment effects in CHF. Vesnarinone is also known to inhibit phosphodiesterase III (PDE3), resulting in an increase in the cyclic AMP concentration in cells, leading to vasodilation (Itoh et al., 1993). Although these pharmacologic effects may be related to the treatment of CHF, the molecular basis of the side effect is not well understood.

Previously, we showed that vesnarinone impairs the production of tumor necrosis factor alpha (TNFα) in bone marrow stromal cells, an event that is essential for the differentiation of the cells into mature granulocytes (Nabeshima et al., 1997; Hiramoto et al., 2004). These finding are also supported by another study that showed that vesnarinone suppressed both the activation of the transcription factor nuclear factor kappa B (NFκB) and the expression of the TNFα gene, a target of NFκB (Manna and Aggarwal 2000).

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ABBREVIATIONS: CHF, congestive heart failure; FG-EDGE, ferrite glycidyl methacrylate-ethylene glycol diglycidyl ether; NFκB, nuclear factor kappa B; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; siRNA, small interfering RNA; TNFα, tumor necrosis factor alpha; VCP, valosin-containing protein.

These findings raised the possibility that the inhibition of NFκB signaling by vesnarinone may cause the observed agranulocytosis.

NFκB is a key transcription factor that regulates many processes, including the immune response, inflammation, and stress responses. When a cell is not stimulated, NFκB is sequestered in the cytosol through the formation of a complex with a member of the IκB family. However, once the cell is stimulated by factors such as TNFα and interleukin (IL)-1β, the IκB kinase phosphorylates IκB, and phosphorylated IκB is then ubiquitinated and degraded by the proteasome. The released NFκB enters the nucleus and functions as a homo- or heterodimer transcription factor with a member of the NFκB family. Genes related to the immune response, inflammation, and other processes are known to be targets of the NFκB transcription factors.

To investigate the mechanism by which vesnarinone inhibits the activation of NFκB, we attempted to purify a vesnarinone-binding protein with high-performance affinity magnetic beads (Shimizu et al., 2000; Nishio et al., 2008), which are powerful tools for the identification of the molecular targets of many drugs, including thalidomide (Ito et al., 2010). Indeed, a valosin-containing protein (VCP) was identified as a result of our application of this method. It is thought that VCP plays important roles in ubiquitin-dependent protein quality control and intracellular signaling pathways [reviewed in Meyer et al. (2012)].

We further showed that VCP is essential for the ubiquitin-dependent proteasome-mediated degradation of IκBα and that vesnarinone induces the accumulation of ubiquitinated IκBα, resulting in the inhibition of NFκB activation by preventing the interaction between VCP and the 26S proteasome.

Materials and Methods

Plasmid Construction, Antibodies, and Materials. Human VCP and IκBα cDNAs were subcloned from a LP101 cell cDNA library into the mammalian expression vector pHyg-EF-2 (Nishizawa et al., 2003). Vectors encoding the VCP deletion mutants were created by polymerase chain reaction (PCR) using mutagenic primers. Antibodies specific for FLAG (M2, mouse monoclonal; Sigma-Aldrich, St. Louis, MO), VCP (mouse monoclonal; Progen, Heidelberg, Germany), IκBα (sc-371, rabbit polyclonal; Santa Cruz Biotechnology, Dallas, TX), phosphorylated IκBα (Cell Signaling Technology, Danvers, MA), actin (EMD Millipore, Billierica, MA), and ubiquitin [FK2, mouse monoclonal; Biomol (Enzo Life Sciences), Farmingdale, NY] were purchased from the indicated suppliers.

Affinity Purification of Vesnarinone-Binding Proteins Using FG-Beads. FG-EGDE (ferrite glycidyl methacrylate-ethylene glycol diglycidyl ether) beads were prepared as described previously (Nishio et al., 2008). The vesnarinone amino acid derivative was incubated with the FG-EGDE beads in distilled water for 24 hours at 37°C. The vesnarinone amino acid derivative-fixed FG-EGDE beads were washed three times with distilled water and stored at 4°C until use. The LP101 cell membrane extracts (Dignam et al., 1983) were incubated with the vesnarinone amino acid derivative-fixed beads for 4 hours at 4°C, and the beads were then washed three times with binding buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 mM EDTA, 10% glycerol, 0.1% NP-40, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml pepstatin A, and 1 μg/ml leupeptin]. The bound proteins were eluted with Laemmli dye or binding buffer containing the vesnarinone amino acid derivative. The eluted proteins were subjected to SDS-PAGE, silver stained, and then subjected to in-gel digestion with trypsin. The peptide fragments were analyzed by quadrupole time-of-flight mass spectrometry, as described previously (Shimizu et al., 2000).

In Vitro Binding Assays. Lysates of 293T cells expressing the VCP mutants were incubated with the vesnarinone amino acid derivative-fixed FG-EGDE for 4 hours at 4°C and washed three times with binding buffer. The bound proteins were eluted with Laemmli dye and subjected to SDS-PAGE, followed by immunoblotting with an anti-FLAG antibody.

Cell Culture, Transfection, and VCP Knockdown. The 293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in 5% CO_2, and the LP101 cells were maintained in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum in 5% CO_2. The knockdown of VCP in the 293T cells was performed using lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA).

Real-Time Reverse Transcription-Polymerase Chain Reaction. The LP101 cells were treated with 5 ng/ml TNFα for 60 minutes, and the 293T cells were treated with 10 ng/ml TNFα for 60 minutes. The total RNAs were then prepared using Sepasol RNA I Super (Nakalai Tesque, http://www.nacalai.co.jp). The quantification of the TNFα or glyceraldehyde-3-phosphate dehydrogenase mRNA levels was performed using the QuantiTect SYBR Green reverse transcription PCR master mix (Qiagen, http://www.qiagen.com).

Coimmunoprecipitation Assay and Immunoblotting. Lipofectamine 2000 was used to transfect the 293T cells with pHyg-IκBα-His-FLAG or pcDNA-VCP-His-FLAG. At 2 days post-transfection, the cells were treated with 5 μM MG132 for 60 minutes, followed by stimulation with 10 ng/ml TNFα for 20 minutes. The cells were then harvested, washed twice with phosphate-buffered saline (PBS), and lysed with radio-immunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 10 mM *N*-ethylmaleimide, 20 mM NaF, 25 μM MG132, 1 mM PMSF, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin] or NP-40 lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% NP-40, 10 mM *N*-ethylmaleimide, 20 mM NaF, 25 μM MG132, 1 mM PMSF, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin] at 4°C for 30 minutes. The samples were centrifuged at 20,000*g* at 4°C for 15 minutes, and the supernatants were incubated with anti-FLAG M2 affinity gel (Sigma-Aldrich) for 2 hours at 4°C. The beads were then washed three times with the same buffer used as the binding buffer, and the bound proteins were eluted with a buffer containing the FLAG peptide (Sigma-Aldrich), subjected to SDS-PAGE, and analyzed by immunoblotting.

Results

Vesnarinone Suppresses the TNFα-Induced Activation of NFκB. Previously, we reported that vesnarinone inhibits the production and the secretion of TNFα from human bone marrow stromal LP101 cells (Nabeshima et al., 1997; Hiramoto et al., 2004), but its molecular mechanisms remained unclear. Based on our previous observations, we first examined whether the inhibition of TNFα secretion in LP101 cells is the result of the reduction of TNFα mRNA expression. After the treatment of LP101 cells with different concentrations of vesnarinone, the cells were induced with TNFα and the quantity of newly transcribed TNFα mRNA was determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR). As shown in Fig. 1A, the expression of TNFα mRNA in the LP101 cells was induced by TNFα and reduced by the addition of vesnarinone in a dose-dependent manner. These results suggested that the observed inhibition of TNFα secretion is the result of decreased TNFα mRNA expression caused by the treatment with vesnarinone.

Next, we used human embryonic kidney 293T cells to examine whether the reduction of TNFα mRNA expression by vesnarinone is cell-type specific. A reduction of the induced

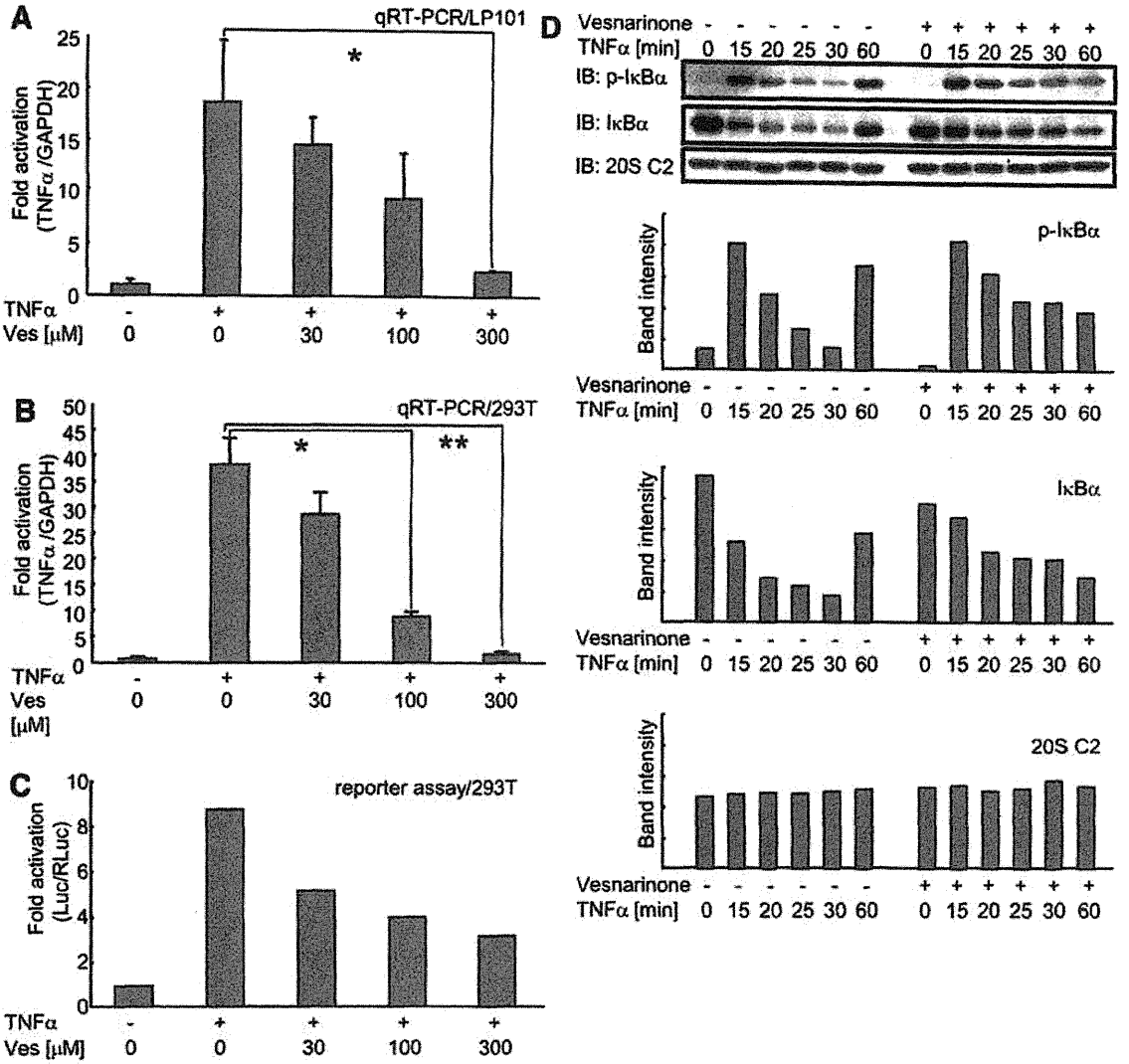

Fig. 1. Vesnarinone inhibits tumor necrosis factor (TNF)α-induced expression of nuclear factor κB target genes. LP101 (A) or 293T (B) cells were preincubated at 37°C for 12 hours with different concentrations (0–300 μM) of vesnarinone, followed by a 60 minutes incubation with or without 10 ng/ml TNFα. The total RNA was prepared and analyzed by reverse transcription Q-PCR using primers specific for TNFα mRNA. The data represent the averages ± S.D. of three independent experiments (*$P < 0.05$). (C) Nuclear factor κB-driven and control reporter plasmids were transfected into 293T cells; 12 hours later, the cells were preincubated with different concentrations (0–300 μM) of vesnarinone for 12 hours, followed by a 12 hours incubation with or without 10 ng/ml TNFα. The cells were harvested, and the cell lysates were subjected to luciferase assays. (D) The 293T cells were preincubated with or without 300 μM vesnarinone for 12 hours and treated with TNFα (10 ng/ml), followed by a 0–60 minutes incubation. The cells were harvested, and IκBα phosphorylation was analyzed by immunoblotting (upper panel). Lower graphs show each band intensity of p-IκB, IαB, or 20S C2.

TNFα mRNA was also observed in this cell line (Fig. 1B), suggesting that the vesnarinone-induced reduction of TNFα expression is not restricted to bone marrow stromal cells.

As NFκB is known to be the major transcription factor regulating TNFα expression, we next investigated whether vesnarinone inhibits NFκB-dependent transcription. A luciferase gene under the control of four tandem-repeated NFκB binding sites was transfected into the 293T cells, and the effects of vesnarinone on the NFκB-driven reporter gene expression after TNFα treatment was examined. The TNFα-induced expression of the reporter gene was inhibited by the treatment with vesnarinone in a dose-dependent manner (Fig. 1C). These results suggested that vesnarinone inhibited NFκB-dependent transcriptional activation.

It is known that NFκB activity is regulated by specific inhibitory subunits, the IκB proteins, which are degraded by the ubiquitin-proteasome system during NFκB activation. If IκB is not properly degraded, NFκB cannot enter the nucleus and fails to activate NFκB-dependent transcription. To determine whether the inhibitory effect of vesnarinone is due to an effect on IκBα degradation, the cytoplasmic level of IκBα protein was measured by immunoblotting (Fig. 1D). In the absence of vesnarinone, a reduction of IκBα protein was observed after 15 minutes of TNFα treatment as expected and the IκBα signal was recovered by 60 minutes. However, in the presence of vesnarinone, the inhibition of IκBα degradation was observed even after TNFα treatment (Fig. 1D). This result suggested that vesnarinone inhibits the IκBα degradation process.

As it is known that the degradation of IκB is triggered by IκB kinase-mediated phosphorylation, we next examined whether IκB is phosphorylated after TNFα treatment. Interestingly,

although the phosphorylation of IκBα was detected either in the presence or absence of vesnarinone at 15 minutes after the TNFα stimulation, the amount of phosphorylated IκBα rapidly decreased in the absence of vesnarinone, which correlated well with the degradation of IκB (Fig. 1D). Conversely, a substantial amount of phosphorylated IκBα was detected even after TNFα stimulation in the presence of vesnarinone. This result correlated well with the remaining amount of IκBα (Fig. 1D). These results suggested that vesnarinone does not inhibit the phosphorylation of IκBα but does inhibit the degradation of IκB at a specific point between IκB phosphorylation and degradation.

Identification of Vesnarinone-Binding Proteins. To clarify the molecular mechanisms of the vesnarinone-induced inhibition of NFκB activation, we attempted to purify vesnarinone-binding proteins directly using high-performance affinity purification (Shimizu et al., 2000). The amino acid derivative of vesnarinone was immobilized on FG beads (Nishio et al., 2008) via the epoxy group (Fig. 2A) and then used for the purification of vesnarinone-binding proteins. The vesnarinone-fixed beads were incubated with extracts of human bone marrow stromal LP101 cells, and the binding proteins were directly purified. A 97-kDa protein was found to bind specifically to the vesnarinone-fixed beads, and the subsequent quadrupole time-of-flight mass spectrometry analysis identified the 97-kDa protein as a valosin-containing protein (VCP) (Fig. 2B). VCP is a member of the ATPases associated with diverse cellular activities (AAA) and possesses two ATPase domains, and it is known to play a critical role in many cellular activities such as the ubiquitin-proteasome system, endoplasmic reticulum-associated degradation of proteins, cell cycle, and DNA repair (reviewed in Meyer et al., 2012). The binding specificity of VCP to vesnarinone was examined by adding free vesnarinone to the elution buffer (Fig. 2B). The addition of free vesnarinone led to the release of VCP from the beads in a concentration-dependent manner, suggesting that VCP is a vesnarinone-binding protein (Fig. 2B). The identification of VCP as a vesnarinone-binding protein was further confirmed by immunoblotting using an anti-VCP antibody (Fig. 2B, bottom), which specifically reacted with the purified 97-kDa protein. In addition, the recombinant VCP (rVCP) protein overexpressed in *Escherichia coli* also has the ability to bind to the vesnarinone-fixed beads, as shown in Fig. 2C, indicating that vesnarinone binds to directly VCP.

Determination of the Vesnarinone-Binding Region of VCP. We then determined the vesnarinone-binding region of VCP. VCP is known to have two ATPase domains, the D1 and D2 domains, which are followed by the N-terminal region of the polyubiquitin-recognition domain (Dai et al., 1998). Thus, we generated a FLAG-His-tagged full-length version and a series of deletion mutants of VCP that have either one or two ATPase domains and assessed the ability of the full-length and mutant recombinant VCP derivatives to bind to the beads. The full-length and deletion mutants consisting of amino acids 186–806, 1–481 and 149–494, which contain the D1 ATPase domain, bound to the vesnarinone-fixed beads. In contrast, two mutants consisting of residues 454–806 and 1–192, lacking the D1 domain, did not bind to the beads (Fig. 3). These results indicated that vesnarinone binds to the central region of VCP, which corresponds to the D1 ATPase domain. This result raised the possibility that VCP is required for NFκB activation and that the binding of vesnarinone to VCP causes a functional alteration in VCP, resulting in the impairment of NFκB activation.

VCP Is Required for NFκB-Dependent Gene Activation. If vesnarinone inhibits VCP function, then the effect of vesnarinone should be similar to that of VCP malfunction. Thus, we knocked down VCP expression using a VCP-specific small interfering RNA (siRNA) and compared the effect of vesnarinone on the transcriptional activation of NFκB target

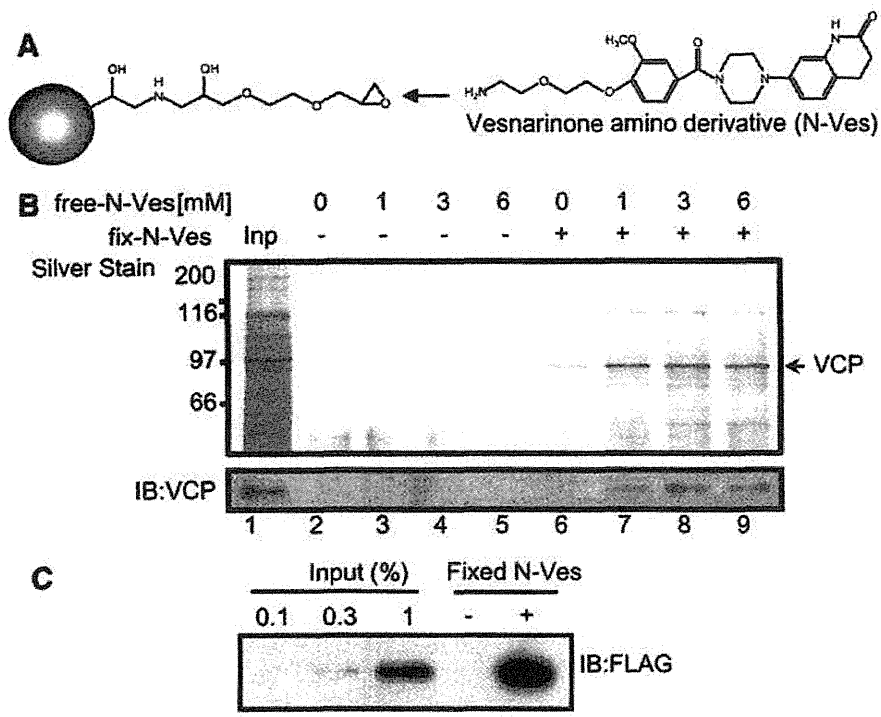

Fig. 2. Identification of VCP as a vesnarinone-binding protein. (A) Structure of the vesnarinone amino acid derivative and FG-beads. (B) Affinity purification of vesnarinone-binding proteins. Vesnarinone-binding protein was purified from LP101 cell extracts with vesnarinone immobilized FG-beads (fix-N-Ves). The bound protein was eluted by increasing concentrations (0–6 mM) of free N-vesnarinone (free-N-Ves). The eluted proteins were analyzed by silver staining (top) and immunoblotting, using an anti-VCP antibody. When the free FG-beads were used (fix-N-Ves), no specific binding protein was purified. (C) Direct binding of vesnarinone to VCP. Recombinant VCP-His-FLAG was incubated with vesnarinone-immobilized FG-beads, and the bound materials were immunoblotted.